differential PKCdelta targeting to multiple myofibrillar proteins other than TnI. Further elucidation of alternative PKCdelta signaling to the myofilments is ongoing. Our results suggest Src-dependent phosphorylation of PKCdelta constitutes an alternative mechanism that allows for stimulus-specific PKCdelta ezymology and myofibrillar targeting within the myocardium.

2580-Pos Board B550

Protein Kinase A-based Modulation Of Ca^{2+} Sensitivity In Skinned Skeletal Muscle Fibers Reconstituted With Cardiac Troponin

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It is well known that protein kinase A (PKA) decreases Ca^{2+} sensitivity in cardiac muscle via phosphorylation of troponin I (TnI). In the present study, we directly tested whether PKA-based phosphorylation of cardiac TnI universally modulates Ca^{2+} sensitivity regardless of the type of muscle, by taking advantage of our Tn exchange technique (Terui et al., J Gen. Physiol. 131;275-283:2008). Troponins were extracted from porcine ventricular and rabbit fast skeletal muscles (Ca^{2+} sensitivity: former < latter). Without Tn exchange, PKA decreased Ca^{2+} sensitivity in cardiac (porcine ventricular) muscle, associated with enhanced phosphorylation of TnI. Reconstitution of cardiac muscle with the skeletal Tn complex (sTn) not only increased Ca^{2+} sensitivity but abolished the PKA effect, suggesting that phosphorylation of TnI, but not of myosin-binding protein C, is primarily responsible for the PKA-based reduction in Ca^{2+} sensitivity. Reconstitution of rabbit psoas muscle with the cardiac Tn complex (cTn) decreased Ca^{2+} sensitivity, as previously reported by us (Terui et al., J Gen. Physiol. 131;275-283:2008). PKA decreased Ca^{2+} sensitivity in cTn-reconstituted skeletal muscle, and subsequent exchange for sTn restored Ca^{2+} sensitivity to the original level. A similar result was obtained when skeletal muscle was reconstituted with the hybrid Tn complex (i.e., cTnI-cTnC-sTnT), suggesting that the troponin I-C complex, but not TnT, is essential for PKA-based modulation of Ca^{2+} sensitivity. These findings support the notion that PKA-based phosphorylation of TnI universally modulates Ca^{2+} sensitivity regardless of the type of muscle.

2581-Pos Board B551

Cardiac Troponin I Threonine 144 phosphorylation: impact on myofilament function

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Threonine 144 (T144) in the inhibitory region of cardiac troponin I (cTnI) is an important site for PKC mediated phosphorylation in the heart. In addition, presence of this residue is sufficient to impart length dependent activation (LDA) properties onto the cardiac sarcomere (Tachampa Circ. Res., 2008). Here we studied the functional impact of a charge mutation so as to mimic phosphorylation of this residue (T144E). Wild-type (WT) or cTnI-T144E containing recombinant troponin (cTn) complexes were exchanged for endogenous cTn in skinned rat cardiac trabeculae. Force and ATPase activity were measured as function of [Ca2+] at short (2.0 μm) or long (2.2 μm) sarcomere length. T144E induced decreased maximum force development (Fmax) and Calcium sensitivity (EC50), increased cross-bridge cycling rate (tension cost) but, in contrast, did not affect LDA (ΔEC50). We conclude that T144E affects cross-bridge cycling and recruitment independent of sarcomere length.

	WT cTn	T144E cTn
EC50 2.0 (μM)	4.0 ± 0.3	5.9 ± 0.4
EC50 2.2 (μM)	3.5 ± 0.2	5.2 ± 0.3
Fmax 2.0 (mN/mm2)	25.1 ± 4.7	13.0 ± 1.7
Fmax 2.2 (mN/mm2)	37.2 ± 4.7	24.5 ± 2.7
Tension cost 2.0	7.7 ± 0.5	12.0 ± 1.3
Tension cost 2.2	6.3 ± 0.5	9.4 ± 1.2
ΔEC50	0.6 ± 0.1	0.7 ± 0.2

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Effects of Cardiac Troponin C Mutants on TnC-TnI interaction and its modulation by PKA phosphorylation

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We are considering several cardiac TnC mutants as potential therapeutic strategies for cardiomyopathies. To judge their potential to improve *in situ* function it is important to understand how these mutants affect TnC-TnI interaction and its modulation by PKA-mediated phosphorylation of TnI Ser 23, Ser 24. In this study, we are characterizing two cardiac TnC mutations, Leu48Glu (L48Q) and Ile61Glu (I61Q), with increased and decreased (respectively) Ca^{2+} binding affinity. In previous studies we showed these mutations resulted in increased (L48Q) and decreased (I61Q) Ca^{2+} sensitivity of steady state force in skinned rat trabeculae. To determine if these changes in Ca^{2+} sensitivity were due to altered TnC-TnI interactions we generated a structural marker by attaching IANBD to Cys84 in the N-lobe of cTnC. Half-maximal IANBD fluorescence saturation of Ca^{2+} binding occurred at pCa7.42 for L48Q cTnC, 7.38 for wild-type (WT) cTnC and 7.30 for I61Q cTnC. In both the absence and presence of saturating Ca^{2+} (0.6 μM TnC) IANBD fluorescence increased with increasing TnI and saturated at different [TnI] in the order L48Q, WT, I61Q. Fluorescence half-maximal saturation occurred at 0.26μM(saturating Ca^{2+}) and 0.25 μM (no Ca^{2+})TnI for L48Q cTnC, 0.78 μM and 0.49μM for WT cTnC , and 1.45 μM and 0.69 μM I61Q cTnC according to the exponential function fit of the data. However, preliminary experiments suggest that when PKA phosphorylated [cTnI] was titrated to cTnC, in both the absence and presence of saturating Ca^{2+}, IANBD fluorescence enhancement with L48Q may be impaired. The data thus far suggest that single amino acid mutations that alter Ca^{2+} binding affinity of TnC can influence interaction with TnI and its modulation by PKA mediated phosphorylation of Ser 23, Ser 24. Supported by HL65497 to MR.

2583-Pos Board B553

Analysis of Cardiac Myofibrillar Troponin I Phosphorylation in Normal and Failing Human Hearts Using Phos-Tags

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Recently, we have used phosphate-affinity SDS-PAGE gels containing Phos-tag-acrylamide (a phosphate-chelating molecule), to determine the level of cardiac troponin I (cTnI) phosphorylation in human myofibrillar extracts. The Phos-tag moiety binds to, and retards, the mobility of phosphoproteins through the gel and results in the separation of the phosphoprotein bands according to their phosphorylation level.

Samples from time-courses of in-vitro PKA catalytic subunit-treated recombinant human cTnI and myofibrillar extracts from non-failing donor, hypertrophic obstructive cardiomyopathy (HOCM) and end-stage failing human heart tissue were analysed by phosphate-affinity SDS-PAGE. Separate gel bands corresponding to 1P, 2P, 3P, 4P and 5P cTnI were observed for the PKA-treated recombinant cTnI. Western blotting probed with the anti-cTnI antibody 14G5 and several different site specific phospho-cTnI antibodies demonstrated that all five of these phospho-species bound to a Ser24P-specific antibody, while a Thr144P-specific antibody only reacted with the 3P, 4P and 5P phospho-species of cTnI.

We observed 3 phospho-species of cTnI in the human heart tissue extracts, which correspond to 0P, 1P and 2P cTnI. Ratios of 0P cTnI were significantly higher in failing and HOCM (both 63 ± 4%) compared to donor (8 ± 2%) while ratios of 2P were significantly lower (failing = 6 ± 2%, HOCM = 8 ± 2%, donor 73 ± 6%). Western blots demonstrated that in human heart cTnI phosphorylation of Ser23/24 was mainly present in the 2P species (with a very small proportion in the 1P species) and that there was no phosphorylation at Thr144. Calculated levels of total cTnI phosphorylation in both HOCM (0.37 ± 0.03, n=50, $p<0.0001$) and failing heart (0.38 ± 0.03, n=24, $p<0.0001$) were significantly reduced from levels in donor heart (1.65 ± 0.04, n=38) and were comparable to previously determined measurements obtained from Pro-Q Diamond phosphoprotein gel staining.

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Effect of Troponin I Ser23/24 Bis-Phosphorylation on Ca^{2+}-Sensitivity is Dependent on PKA Phosphorylation of Other Contractile Proteins

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Upon β-adrenergic stimulation, protein kinase A (PKA) enhances cardiac Troponin I (cTnI) phosphorylation at ser23/24. PKA treatment leads to a decrease in myofilament Ca^{2+}-sensitivity. However, the specific effect of PKA-mediated phosphorylation of cTnI in human myocardium is unclear since PKA phosphorylates a broader set of contractile proteins, in particular myosin binding protein C (cMyBP-C).

To address this issue, a selective exchange procedure was used in which 50% and 70% of the endogenous cTn complex in permeabilized human cardiomyocytes was exchanged with recombinant unphosphorylated human cTn. Cardiomyocytes isolated from healthy donor hearts showed almost saturated phosphorylation levels at the ser23/24 of cTnI. Endogenous phosphorylated cTn of donor cardiomyocytes (pCa_{50}= 5.45 ± 0.03) was exchanged with 0.5 and 1.0 mg/ml unphosphorylated recombinant human cTn (cTn-U), which resulted in an increase in Ca^{2+}-sensitivity (ΔpCa_{50}=0.08). Subsequent incubation of the cells with PKA reversed Ca^{2+}-sensitivity to baseline levels (pCa_{50}= 5.46 ± 0.03).

To study if the effect of PKA-mediated phosphorylation on cTnI ser23/24 depends on phosphorylation of other contractile proteins, failing human cardiac tissue was used in which phosphorylation of cTnI and cMyBP-C is depressed. Cells from failing tissue showed increased Ca^{2+}-sensitivity (pCa_{50} 5.56 ± 0.03) compared to donor cells. Endogenous cTn of failing cardiomyocytes was exchanged with 0.5 and 1.0 mg/ml cTn pre-treated with PKA to fully saturate ser23/24 (cTn-bisP). However, upon exchange with the cTn-bisP complex, Ca^{2+}-sensitivity did not decrease. Subsequent PKA incubation reduced pCa_{50} back to the level observed in donor myocardium. This indicates that the effect of cTnI ser23/24 bis-phosphorylation on Ca^{2+}-sensitivity is dependent on PKA-mediated phosphorylation of other contractile protein(s). Preliminary protein phosphorylation data point towards the involvement of cMyBP-C.

2585-Pos Board B555

EM and 3D-Reconstruction of Thin Filaments Reconstituted with Truncated Troponin I Associated with Myocardial Stunning

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Myocardial ''stunning'', a condition resulting from a short period of ischemia followed by reperfusion, can cause cardiac dysfunction, despite the absence of tissue necrosis. Stunning can be associated with rapid proteolytic truncation of the C-terminal 17 acids of TnI (to form ''$TnI_{1\text{-}192}$''). Expression of $TnI_{1\text{-}192}$ in transgenic mice is sufficient to account for the stunning phenotype (Murphy *et al.*, 2000), where for example myofibrils containing $TnI_{1\text{-}192}$ and otherwise normal troponin-tropomyosin display increased Ca^{2+}-sensitivity (Narolska *et al.*, 2006; also Foster *et al.*, 2003). In the current study, electron microscopy and 3D-image reconstruction of thin filaments containing $cTnI_{1\text{-}192}$ and control TnC, TnT and tropomyosin was performed to determine if the truncation causes an imbalance in the tropomyosin distribution between different regulatory states. Negatively stained ''mutant'' filaments showed characteristic periodic troponin projections and tropomyosin strands. Both helical reconstruction and single particle analysis indicated that at low-Ca^{2+} the tropomyosin localized on the inner aspect of the outer domain of actin. As expected, tropomyosin moves to the inner domain of actin in Ca^{2+} (Foster *et al.*, 2003). However, truncated TnI appears to promote an extra transition of tropomyosin from the Ca^{2+}- induced, closed position on actin toward the myosin-induced, open-state position. Here, tropomyosin in myosin-free thin filaments appears biased towards the open-state in the presence of only Ca^{2+}. Cross-correlation of filament segments to models of the blocked-, closed-, and open-states (as in Pirani *et al.*, 2005) confirms this open-state bias, which correlates well with the increase in Ca^{2+}-sensitivity observed in *in vitro* and in fiber assays of function.

2586-Pos Board B556

Impact Of N-terminal Truncation Of Cardiac Troponin I On Myofilament Chemo-mechanical Transduction: Implications For The Enhanced Cardiac Function In Hemodynamic Adaptation

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The deletion of N-terminal extension of cardiac troponin I($cTnI_{ND}$) by restricted proteolysis has been recently proposed to be a novel mechanism to regulate cardiac function during hemodynamic adaptation. In vivo and isolated working heart from transgenic mice overexpressing $cTnI_{ND}$ revealed an enhanced rate of relaxation and reduced end diastolic pressure. However, the functional effect of $cTnI_{ND}$ on myofilament properties has not been fully evaluated. Accordingly, we determined the functional effects of $cTnI_{ND}$ on cardiac tension cost(cross-bridge cycling), maximal tension development(F-max) and Ca^{2+}-sensitivity(EC_{50}) using mechanical force- and enzyme-coupled UV absorbance measurements. Wild-type(WT) or $cTnI_{ND}$ containing recombinant troponin(cTn) complexes were exchanged for endogenous cTn in skinned rat cardiac trabeculae. $cTnI_{ND}$ induced a significantly reduction in Fmax and Ca^{2+}-sensitivity but increased cross-bridge cycling rate. In addition, by using steady-state fluorescence measurements, we found that the decreased myofilament Ca^{2+} sensitivity is due to a decrease in Ca^{2+} binding affinity of the regulatory site of cTnC in the thin filament. We conclude that increased cross-bridge cycling rate by $cTnI_{ND}$ may underlie, in part, the modulation of cardiac function and hemodynamic adaptation associated with $cTnI_{ND}$.

Summarized Table

	WT (N=10)	cTnIND (N=8)	p value
Fmax (mN/mm2)	42.9 ± 5.1	17.8 ± 1.7	0.0007*
Hill	4.1 ± 0.7	5.1 ± 0.6	0.2587
EC50 (uM)	2.6 ± 0.2	3.7 ± 0.2	0.0022*
Tension Cost	7.5 ± 0.6	11.4 ± 1.3	0.0140*

2587-Pos Board B557

Structural and Proteomic Analysis of the Drosophila Cardiac Tube

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Drosophila melanogaster possess a simple linear heart tube which is an efficient in vivo system for studying basic developmental and physiological processes as well as for investigating potentially conserved pathogenic mechanisms of genetically inherited cardiac disorders. Human cardiomyopathies commonly arise from cytoarchitectural mutations. We previously showed that, as in humans, Drosophila exhibit disparate cardiac responses to depressed or enhanced myosin biomechanical properties. Here, we further characterized the morphological and ultrastructural consequences of altered myosin function on the Drosophila heart. Fluorescent microscopy revealed D45 flies, expressing myosin with depressed ATPase and in vitro sliding properties, show cardiac dilation with relatively normal myofibrillar organization. However, Mhc5 fly hearts, expressing myosin with enhanced molecular properties show centrally located restricted regions, a loss of contractile material and myofibrillar disarray. Moreover, electron microscopy revealed perturbed sarcomeric organization of the cardiomyocytes in both mutants. Mitochondria appeared swollen with apparent increased matrix volume and membranic rupture resulting in a prevalence of vacuolization. These cardiac phenotypes bear similarity to those observed in human cardiomyopathies and imply the existence of conserved pathological responses to altered myosin motor function. To further substantiate the use of the Drosophila heart as a model for investigating developmental, physiological and pathological processes and to identify conserved and potentially unique molecular components, we have undertaken preliminary proteomic analysis of isolated hearts. LC-MS/MS analysis identified ~450 proteins with high confidence. The cardiac proteins derive primarily from the sarcomere, cytoskeleton and the mitochondria. Many of the major cardiac components appear conserved between flies and humans. We ultimately seek to use quantitative proteomic studies to identify how specific lesions of myosin perturb protein networks within the Drosophila heart, and to determine how these perturbations contribute to the pathogenesis of cardiomyopathy.

2588-Pos Board B558

In-Solution Proteomic Workflow for Purification of Endogenous Sarcomeric Proteins and Identification of Distinct Charged Variants of Regulatory Light Chain

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The molecular conformation of the myosin motor is modulated by intermolecular interactions with the light chains, C-protein and titin, and governed by post-translational modifications (PTMs). These PTMs are important in regulation of function in ejecting ventricles as mechanisms downstream of Ca^{2+} fluxes at the level of the sarcomere appear to dominate ejection and sustain